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Characterization of Epoxydecenal Isomers as Potent Odorants in Black Tea (Dimbula) Infusion

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In a black tea (Dimbula) infusion, the potent "sweet and/or juicy" odorants were identified as the *cis*- and *trans*-4,5-epoxy-(*E*)-2-decenals by comparison of their gas chromatography retention indices, mass spectra, and odor quality to those of the actual synthetic compounds. Of the two odorants, *cis*-4,5-epoxy-(*E*)-2-decenal has been identified for the first time in the black tea. On the basis of the aroma extract dilution analysis on the flavor distillate obtained using the solvent-assisted flavor evaporation technique from the black tea infusion, these isomers showed higher flavor dilution (FD) factors. The FD factors and concentrations of these odorants in the black tea infusion were observed to be much higher than those from Japanese green tea. In addition, the model studies showed that these odorants were generated from linoleic acid and its hydroperoxides by heating, but the generated amounts of these odorants from linoleic acid were much less than those of its hydroperoxides. It can be assumed from these results that the withering and fermentation, which are characteristic processes during the manufacturing of the black tea, which includes the enzymatic reaction such as lipoxygenase, is one of the most important factors for the formation of the epoxydecenal isomers.

KEYWORDS: Dimbula; black tea; *cis*-4,5-epoxy-(*E*)-2-decenal; *trans*-4,5-epoxy-(*E*)-2-decenal; manufacturing process; enzyme reaction

INTRODUCTION

Black tea is one of the most widely consumed beverages in the world. The high acceptability of black tea is due to many factors, one of the most important being its flavor. Black tea is produced in many places around the world, and many cultivars are known. These cultivars have characteristic flavors, such as the Darjeeling muscat flavor and Keemun smoky flavor, due to various primary factors, for instance, the climate, soil, manufacturing conditions, etc. On the other hand, the so-called Ceylon black teas, which are produced in Sri Lanka, have a typical floral and juicy−sweet note and have been preferred by the Japanese for a long time.

The black tea flavor has already been the subject of much research, and many of the volatile compounds have been identified (*1*). A number of volatile components in the Ceylon black tea flavor have also been identified by gas chromatography (GC) and GC−mass spectrometry (MS) measurements (*2*−*4*). However, there are few reports concerning the potent odorants of the black tea flavor (*5*−*9*). In these reports, many potent odorants have been identified, and the flavor difference of each cultivar is assumed to be caused by the difference in the amount of the potent odorants (*6*, *8*). However, there is no report about the potent odorants of the Ceylon black tea, and the contributors to the typical flavor of this black tea have not been identified, because the previous flavor analysis of the Ceylon black tea was not coupled with an aroma extract dilution analysis (AEDA).

The steps involved in the processing of black tea include withering, leaf disruption (rolling and/or cutting), fermentation, drying, and grading. For the characteristic process during the black tea production, it utilizes an enzyme reaction. Namely, black tea leaves were produced by drying after making use of the sufficient enzyme action during the withering and fermentation process. On the other hand, the green tea leaves inactivated the enzyme by steaming or parching during the first process, and it hardly utilized the action of the enzyme. The black tea flavor was quite different from that of the green tea regardless of being made from the same kind of plant. For the flavor formation of the black tea, in particular, it was pointed out that the enzyme action during the manufacturing process is very important for the intense monoterpene alcohols with glycosidase (*10*, *11*), aldehydes with polyphenol oxidase (*12*, *13*), and C6 aldehydes with lipoxygenase and hydroperoxide lyase (*14*). However, the influence of the enzyme action during the manufacturing process on the formation of the potent odorants is still mostly unresolved.

The objective of the present investigation was to elucidate the potent odorants of Dimbula, which is one of the typical Ceylon black teas by an AEDA. Furthermore, to clarify the generation mechanism of the epoxydecenal isomers during the manufacturing of the black tea leaves, a comparison of the black

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and green teas and model experiments using the linoleic acid and its hydroperoxides were performed.

MATERIALS AND METHODS

Materials. *Tea Samples.* The black tea products are as follows: Dimbula (Sri Lanka), Uva (Sri Lanka), Nuwara eliya (Sri Lanka), Darjeeling (India), Assam (India), Java (Indonesia), and Keemun (China). These black tea products were produced in 2004, and they were purchased from Mitsui Norin Co., Ltd. (Tokyo, Japan). The Japanese green tea (Sen-cha) product was produced in Shizuoka prefecture (Japan) in 2004. These black and green tea leaves were stored at −80 °C until needed.

Chemicals. (*E*,*E*)-2,4-hexadienal, (*E*,*E*)-2,4-heptadienal, (*E*,*E*)-2,4-octadienal, (*E*,*E*)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal, linalool, *n*-hexanoic acid, and vanillin were obtained from Tokyo Kasei Kogyo (Tokyo, Japan), β-damascenone was obtained from Nihon Firmenich (Tokyo, Japan), and linoleic acid, grycerol trioctanoate, and soy bean lipoxygnase were obtained from Wako Pure Chemical Industries (Osaka, Japan).

Tea Infusion. Deionized hot water (3 L) at 85−90 (black tea) or 70−75 °C (green tea) was added to 150 g of tea, and the leaves were removed using coarse filter paper after the mixture stood for 5 min. The filtrate (3 L) was immediately cooled to about 20 °C in tap water.

Syntheses of *cis*-4,5-Epoxy-(*E*)-2-decenal and *trans*-4,5-Epoxy-(*E*)-2-decenal. The target compounds were synthesized from *cis*- or *trans*-2-octenol as the starting materials by epoxidation with *m*-chloroperbenzoic acid (*m*-CPBA), followed by oxidation with Dess−Martin periodinane and the Wittig reaction (*15*). In the first step, *cis*- or *trans*-2,3-epoxyoctanol was synthesized using *m*-CPBA. The *m*-CPBA (purity > 65%, 31.9 g) was then dissolved in methylene chloride (500 mL), and this solution was dropped into (*Z*)-2-octenol (purity > 95%, 0.1 mol, Eiweiss Chemical Corp., Shizuoka, Japan) or (*E*)-2-octenol (purity > 95%, 0.1 mol, Sigma-Aldrich Japan, Tokyo, Japan) solution, which was dissolved in methylene chloride (1000 mL) at 0−5 °C, and the mixture was stirred for 2 h at room temperature. When the epoxidation stopped, saturated solutions of $Na_2S_2O_3$ and $NaHCO_3$ were added and then extracted with Et_2O (3 × 100 mL). The combined organic phases were washed with a saturated solution of NaCl (2 × 100 mL) and then dried over anhydrous sodium sulfate. After the solvent was removed by evaporation, the compounds were purified by column chromatography on silica gel, and then, 13.5 g of *cis*-2,3-epoxyoctanol or 13.5 g of *trans*-2,3-epoxyoctanol, each with a purity of >97%, was obtained. The obtained *cis*- or *trans*-2,3-epoxyoctanol was then oxidized into the corresponding aldehyde, *cis*- or *trans*-2,3-epoxyoctanal, using Dess−Martin periodinane. The *cis*- or *trans*-2,3-epoxyoctanol (2.9 g) was dissolved in methylene chloride (300 mL), and then, a 15%-Dess−Martin periodinane solution (dissolved in methylene chloride, 85 g) was added, and the solution was stirred for 2 h at room temperature. When the oxidation stopped, saturated solutions of $Na_2S_2O_3$ and $NaHCO_3$ were added and then extracted with Et_2O (3 × 100 mL). The combined organic phases were washed with a saturated solution of NaCl (2 × 100 mL) and then dried over anhydrous sodium sulfate. After the solvent was removed by evaporation, the compounds were purified by column chromatography on silica gel; then, 2.4 g of *cis*-2,3-epoxyoctanal or 2.5 g of *trans*-2,3-epoxyoctanal, each with a purity of >97%, was obtained. In the third step, the target compounds were obtained by the Wittig reaction, in which the *cis*-2,3-epoxyoctanal (2.4 g) or *trans*-2,3-epoxyoctanal (2.4 g) and formylmethylene triphenylphosphorane (5.2 g) were dissolved in toluene (110 mL), and the reaction mixture was refluxed for 8 h. After it was cooled, the reaction mixture was evaporated to remove the solvent, and the residue was extracted with hexane. After the solvent was removed by evaporation, the compounds were purified by column chromatography on silica gel; then, 1.7 g of *cis*-4,5-epoxy-(*E*)-2-decenal or 1.8 g of *trans*-4,5-epoxy-(*E*)-2-decenal, each with a purity of > 97%, was obtained. These structures were confirmed by mass spectrometry (electron impact [EI] mode) and ^{1}H nuclear magnetic resonance. The ^{1}H NMR characterization afforded the following data: δ [multiplicity, coupling constant (in hertz), and relevant H at carbon (numbering refers to **Figure 3**)]. *trans*-4,5-Epoxy-(*E*)-2-decenal: MS/EI *m*/*z* (%) 152 (2, [M^+]), 139 (4), 81 (27), 69 (11), 68 (100), 55 (15), 43 (12), 41 (20), 39 (18). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.92 (t, J = 7.1 Hz, 3H, C-10), 1.32−1.36 (m, 4H, C-8,9), 1.49 (m, 2H, C-7), 1.64−1.66 (m, 2H, C-6), 2.96 (td, J_{5-6} = 5.6 Hz, J_{5-4} = 2.0 Hz, 1H, C-5), 3.33 (dd, J_{4-3} = 7 Hz, J_{4-5} = 2 Hz, 1H, C-4), 6.38 (dd, J_{2-1} = 7.6 Hz, J_{2-3} = 15.8 Hz, 1H, C-2), 6.56 (dd, J_{3-4} = 7 Hz, J_{3-2} = 15.8 Hz, 1H, C-3), 9.56 (d, J_{1-2} = 7.6 Hz, 1H, C-1). *cis*-4,5-Epoxy-(*E*)-2-decenal: MS/EI *m*/*z* (%) 152 (3, [M^+]), 139 (6), 81 (27), 69 (11), 68 (100), 55 (16), 43 (12), 41 (17), 39 (22). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 0.90 (t, J = 7.1 Hz, 3H, C-10), 1.23−1.45 (m, 4H, C-8,9), 1.45−1.65 (m, 4H, C-6,7), 3.28 (m, 1H, C-5), 3.63 (ddd, J_{4-3} = 6.5 Hz, J_{4-5} = 4.5 Hz, J_{4-2} = 0.9 Hz 1H, C-4), 6.40 (ddd, J_{2-1} = 7.8 Hz, J_{2-3} = 15.8 Hz, J_{2-4} = 0.9 Hz 1H, C-2), 6.69 (dd, J_{3-4} = 6.5 Hz, J_{3-2} = 15.8 Hz, 1H, C-3), 9.60 (d, J_{1-2} = 7.8 Hz, 1H, C-1).

Isolation of the Volatiles from Tea Infusion. Each tea (black and green) infusion (1 L) was passed through a column packed with 10 g of Porapak Q (80/100 mesh, Waters). The adsorbed compounds were then eluted with 100 mL of methylene chloride. The eluate was dried over anhydrous sodium sulfate (approximately 10 g), and to remove the nonvolatile material, the eluate was distilled under reduced pressure (40 °C at 5 × 10^{-3} Pa) using the solvent-assisted flavor evaporation (SAFE) method (*16*). The distillate was dried over anhydrous sodium sulfate, and the solvent was then removed by rotary evaporation (35 °C at 550 mmHg) to about 5 mL. Further concentration was conducted in a nitrogen stream to about 150 μL. For the quantitative analysis, an internal standard solution (10 μL) prepared from methyl undecanoate (5.06 mg) in methylene chloride (10 mL) was added to the eluate before the SAFE treatment, and then, the acids in the tea distillate were removed using a saturated solution of $NaHCO_3$ (2 × 50 mL), because the epoxydecenal isomer peaks were overlapped with the hexenoic acid peaks on the gas chromatogram using the DB-Wax stationary phase column. The combined organic phases were washed with a saturated solution of NaCl (2 × 100 mL) and then dried over anhydrous sodium sulfate. The resulting concentrate was used as the sample for the AEDA and the GC-MS analysis.

Enrichment of Epoxydecenal Isomers for Identification. For the identification experiments, the black tea volatiles were isolated from the black tea infusion (Dimbula) by combining the adsorptive column method and the SAFE technique as described above. These procedures were repeated, and all of the volatile fractions were combined (total: 6 L of deionized water was added to 450 g of black tea leaves). The concentrated volatile fraction was applied to a water-cooled glass column (15 °C, 20 cm × 0.7 cm i.d.) filled with silica gel (wakogel C-200; Wako Pure Chemical Industries) in isopentane. After the column had been flushed with isopentane (100 mL) and isopentane/methylene chloride (150 mL, 80/20, v/v), the epoxydecenal isomers were eluted with methylene chloride (100 mL). The solution was concentrated to about 100 μL as already described.

Preparation of Linoleic Acid Hydroperoxides. Linoleic acid hydroperoxides were obtained from the oxidation of linoleic acid by soybean lipoxygenase. The hydroperoxides were isolated by column chromatography and used as soon as possible to avoid the model reaction. Linoleic acid (1 g) was emulsified with 0.8 mL of 0.1% Tween 80 in 500 mL of 20 mmol sodium borate buffer (pH 8.5). After saturation with oxygen for 10 min, the substrate was incubated at room temperature with 20 mg of lipoxygenase dissolved in 1 mL of the same buffer. After 1 h, the mixture was acidified with concentrated HCl to pH 3 and extracted with 3 × 250 mL of methylene chloride. The combined organic phases were washed with distilled water (2 × 250 mL) and then dried over anhydrous sodium sulfate. The oxidation product of linoleic acid by soybean lipoxygenase was subjected to thin-layer chromatography (TLC) using Silica Gel 60 F_{254} plates (Merck Japan Ltd., Tokyo, Japan). After two developments with the solvent system, hexane, diethyl ether, and formic acid (50 + 50 + 1), the hydroperoxides were detected on the TLC plates by UV irradiation, and the major product gave the R_f of 0.54. After the solvent was removed by evaporation, the reaction mixture was dissolved in about 2 mL of hexane and then purified by column chromatography on silica gel. The reaction mixture, dissolved in hexane, was placed on the top of a column (2 cm i.d.) packed with a slurry of 50 g silica gel (Wakogel C-200) in hexane. Elution was performed using the following sol-

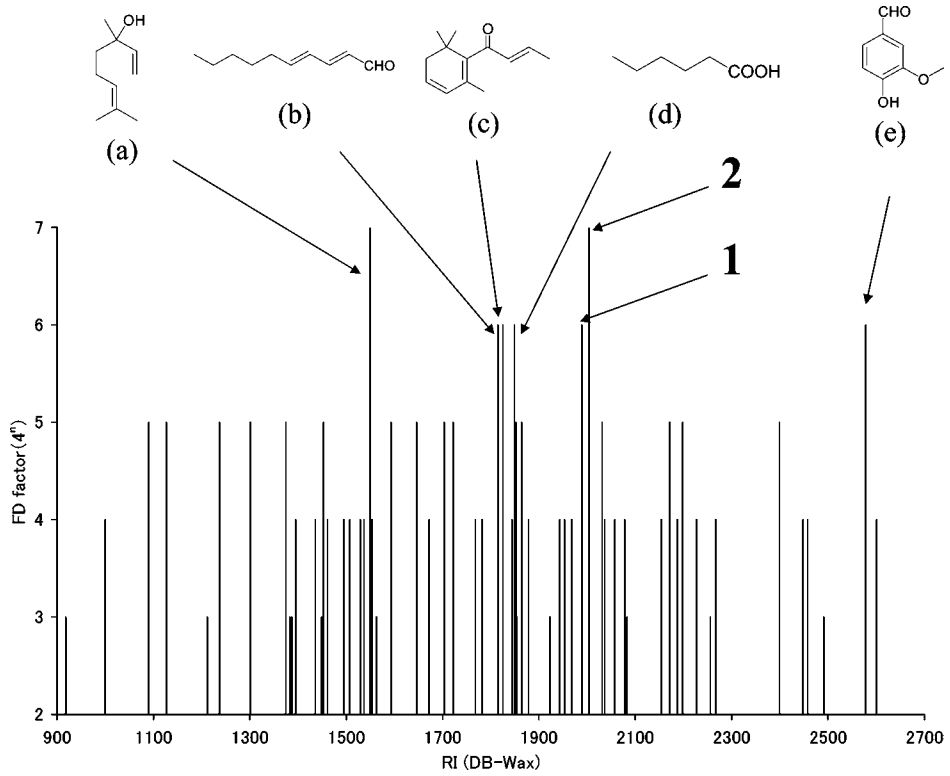

Figure 1. Flavor dilution chromatograms of the black tea (Dimbula) infusions (**a**, linalool; **b**, (*E*,*E*)-2,4-decadienal; **c**, β-damascenone; **d**, hexanoic acid; and **e**, vanillin).

vents: hexane (100 mL), hexane/diethyl ether (8/2, 100 mL), hexane/diethyl ether (7/3, 100 mL), hexane/diethyl ether (6/4, 100 mL), and hexane/diethyl ether (1/1, 100 mL). The separated fractions (30 mL each) were monitored for hydroperoxides by UV absorption on the TLC plates, and the major product was collected. Immediately after the solvent was removed by rotary evaporation, 380 mg of the linoleic acid hydroperoxides was dissolved in 10 mL of methylene chloride.

Model Experiment for Epoxydecenal Isomer Formation from Linolenic Acid. The linoleic acid hydroperoxides (31.25 μmol) or linoleic acid (31.25 μmol) was dissolved in 5 g of glycerol trioctanoate containing $FeSO_4 \cdot 2H_2O$ (2 mg) and then heated for 60 min at 150 °C using a GC oven in a closed vessel. After the mixture was cooled, methylene chloride (50 mL), containing methyl undecanoate (101 μg) as the internal standard, was added, and the flavor compounds and internal standard were isolated by the SAFE treatment. The epoxydecenal isomers were then quantified by GC-MS.

Gas Chromatography−Olfactometry (GC-O). A Hewlett-Packard (HP) 5890 series gas chromatograph equipped with a thermal conductivity detector (TCD) and fused silica column (30 m × 0.25 mm i.d., coated with a 0.25 μm film of DB-Wax; J & W Scientific) was used in the splitless injection mode (splitless time, 1 min). The column temperature was programmed from 40 to 210 °C at the rate of 5 °C/min for all runs. The injector and detector temperatures were 250 and 230 °C, respectively. Helium was used as the carrier gas at the flow rate of 1 mL/min. A glass sniffing port was connected to the outlet of the TCD and heated by a ribbon heater with moist air being pumped into the sniffing port at about 100 mL/min to quickly remove the odorant from the sniffing port that had been eluted from the TCD.

AEDA. The original odor concentrate of the tea infusion was stepwise diluted with methylene chloride to 4^n ($n = 3-8$), and aliquots (1 μL) of each fraction were analyzed by capillary GC using a DB-Wax column. The odorants were then detected by GC eluate sniffing (GC-O). The flavor dilution (FD) factors of the odorants were determined by AEDA (*17*). Before the FD factor measurement, two panelists repeatedly checked the retention time and the odor quality of the odorants using each diluted sample (1:64), and then, the FD factor of the odorants was determined by being detected at the dilution step by both panelists.

Table 1. Selected Ion and Calibration Factors for Mass Chromatography (SIM)

compound	selected ion (*m*/*z*)	calibration factor
(*E*,*E*)-2,4-hexadienal	96	0.22
(*E*,*E*)-2,4-heptadienal	110	0.28
(*E*,*E*)-2,4-octadienal	124	0.34
(*E*,*E*)-2,4-nonadienal	138	0.45
(*E*,*E*)-2,4-decadienal	152	0.58
cis-4,5-epoxy-(*E*)-2-decenal	68	0.10
trans-4,5-epoxy-(*E*)-2-decenal	68	0.08
methyl undecanoate[a]	200	1

[a] Internal standard.

GC-MS. An Agilent 6890 N gas chromatograph coupled to an Agilent 5973 N series mass spectrometer was used. The column was a 60 m × 0.25 mm i.d. DB-Wax fused silica capillary type (J & W Scientific) with a film thickness of 0.25 μm. The column temperature was programmed from 80 to 210 °C or from 40 to 210 °C at the rate of 3 °C/min. The injector temperature was 250 °C, and the flow rate of the helium carrier gas was 1 mL/min. An injection volume of 1 or 0.2 μL was applied using the split (the split ratio was 1:30) or splitless technique. The mass spectrometer was used with an ionization voltage of 70 eV (EI) and an ion source temperature of 150 °C. The quantities of the components in each volatile fraction of the tea infusions were determined from the extracted ion peak areas obtained by mass chromatography. The GC-MS was operated in the selected ion mode (SIM), and the extracted ions were monitored in the ranges listed in **Table 1**. The calibration factors were determined in a mixture of equal amounts by weight of an odorant and internal standard compound and were calculated as the ratio of the extracted ion peak area of the internal standard to the extracted ion peak area of the odorant. These extracted ion peak areas were the mean values of the triplicate results. The calibration factors were used to calculate the amount of each odorant on the basis of the internal standard.

Identification of the Components. Each component was identified by comparing its Kovats GC retention index (RI), mass spectrum, and odor quality with those of an authentic compound.

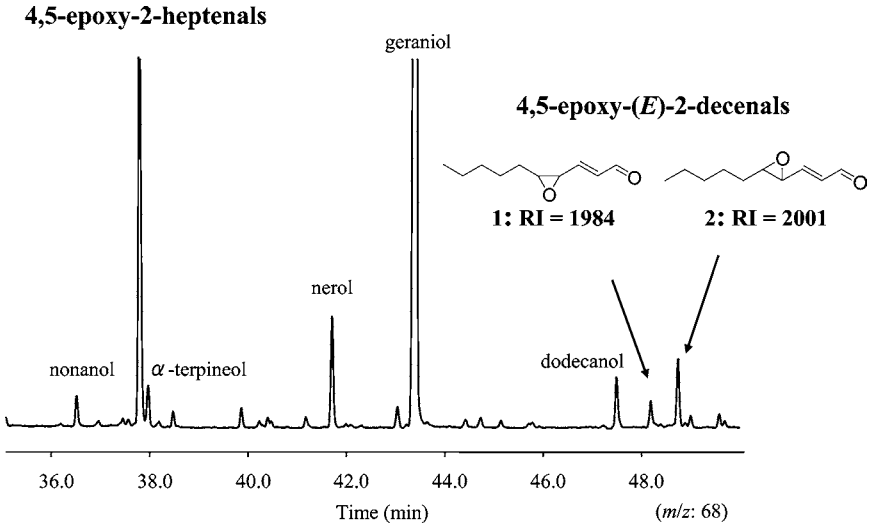

Figure 2. Mass chromatogram of the volatile concentrate of a black tea (Dimbula) infusion showing the extracted ion, which is the typical fragment ion of the homologous series of 4,5-epoxy-2-alkenals [peak 1, *cis*-4,5-epoxy-(*E*)-2-decenal; peak 2, *trans*-4,5-epoxy-(*E*)-2-decenal].

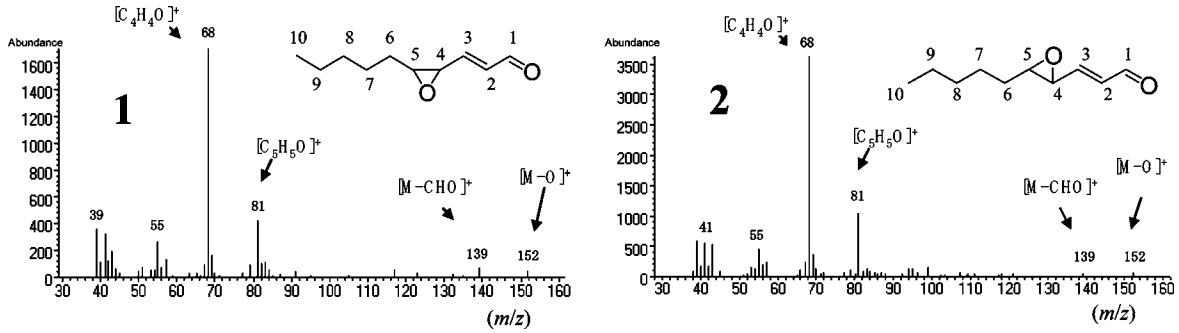

Figure 3. Mass spectra of *cis*-4,5-epoxy-(*E*)-2-decenal (**1**) and *trans*-4,5-epoxy-(*E*)-2-decenal (**2**), which were obtained from the enriched fraction.

Proton Magnetic Resonance Spectrometry (^{1}H NMR). The ^{1}H NMR spectra were recorded in $CDCl_3$ solution using a Bruker AVANCE 400 spectrometer operating at 400 MHz and using tetramethylsilane as the internal standard.

RESULTS AND DISCUSSION

Potent Odorants in Ceylon Black Tea Flavor. The flavor extract of the Ceylon black tea infusion was prepared by combining the adsorptive column method and the SAFE technique. The flavor extract well-reproduced the characteristic odor of the Ceylon black tea infusions. The AEDA applied to the volatile fraction, which had been prepared from freshly filtered Ceylon black tea infusion, revealed 61 odor active peaks with FD factors between 4^3 and 4^7 (**Figure 1**). Among the perceived odorants, seven peaks whose higher FD factors ($\geq 4^6$) have been proved to be the most important components of the Ceylon black tea flavor, and these were identified by comparing their Kovats indices, mass spectra, and odor quality with those of the authentic compounds, as detailed in **Figure 1**. Five compounds were identified from seven peaks by GC-MS [**a**, linalool (floral); **b**, (*E*,*E*)-2,4-decadienal (fatty); **c**, *β*-damascenone (sweet); **d**, hexanoic acid (acidity); and **e**, vanillin (vanilla-like)], and these odorants have been shown to be some of the most important compounds for the other kinds of black tea cultivars (*5*−*9*). In **Figure 2**, the mass chromatogram of the volatile concentrate of a Ceylon black tea infusion shows the extracted ion with *m*/*z* 68. Peaks 1 (RI DBWax = 1984) and 2 (RI DBWax = 2001) with the characteristic juicy and sweet notes were identified as 4,5-epoxy-2-decenals by their mass spectra obtained from the enriched fraction (**Figure 3**). *trans*-4,5-Epoxy-(*E*)-2-decenal (**2**) has already been found to be a potent odorant of many foodstuffs (*18*−*20*), and peak 2 can be presumed to correspond to **2** by comparison of the retention index in the literature (*21*). The mass spectrum of **2** gave typical fragment ions derived from its structure. On the other hand, peak 1 also gave similar fragment ions as did peak 2. Therefore, peak 1 is thought to be an isomer of **2** and is assumed to be *cis*-4,5-epoxy-(*E*)-2-decenal (**1**).

To confirm the structures of **1** and **2**, both epoxy isomers were synthesized, and identification of the odorants in the black tea was confirmed by comparison of their retention indices. The geometric structure of the epoxy and double bond part of the synthesized **1** and **2** was verified using the ^{1}H NMR coupling constant, and the structure of these isomers was characterized as *cis*-epoxy, *trans*-ene (**1**) and *trans*-epoxy, *trans*-ene (**2**). The retention indices of each of the 4,5-epoxy-(*E*)-2-decenals were calculated by GC and compared to the peaks of the black tea. As a result, the synthesized **1** and **2** closely agreed with peaks 1 and 2 in the volatile fraction of the black tea infusion. Of the two odorants, **1** is reported here for the first time as a component of the black tea flavor. Odorant **1** was first identified in fresh field tomatoes (*22*, *23*); however, this epoxydecenal has not yet been identified in other kinds of aromas arising from natural sources. Each odorant (**1**, **2**) has a common juicy, sweet, and metallic note. However, in addition to the common note, **1** has a fatty note, and **2** has a fresh citrus-like note; thus, the odor of these isomers slightly differed. **Figure 4** shows the amounts of the epoxydecenal isomers in the different black tea cultivars. These data indicated that the contents of **1** and **2** differ in every cultivar, and the amount of these odorants in Dimbula was more

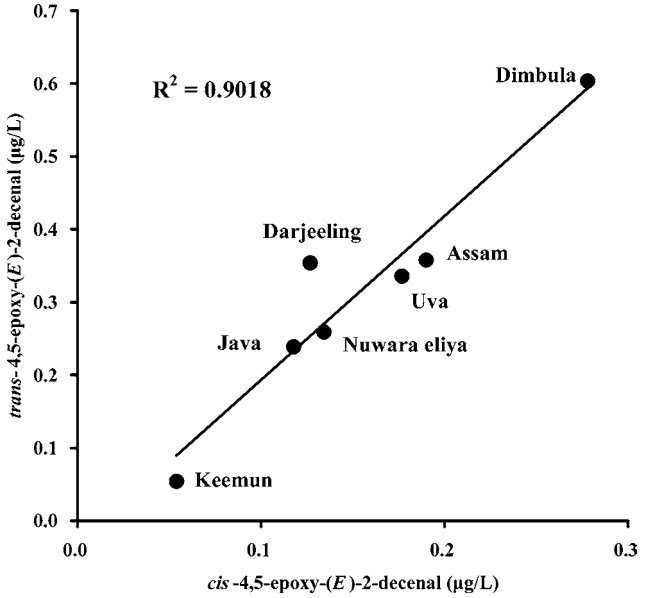

Figure 4. Amounts of 4,5-epoxy-(*E*)-2-decenal isomers in the different black tea cultivars.

than in the other kinds of black teas. Therefore, the epoxydecenal isomers (**1**, **2**) seem to be responsible for the characteristic flavor of the Ceylon black tea, especially the juicy and sweet note.

Formation Mechanism of Epoxydecenal Isomers in the Black Tea. *trans*-4,5-Epoxy-(*E*)-2-decenal (**2**) has been proposed to originate from linoleic acid by oxidation (*24*, *25*). It has been reported that the tea leaves contain from 3 to 5% fatty acids, and linoleic acid is one of the principal fatty acids (*26*). Therefore, **2** in black tea would be expected to be generated from the linoleic acid in the tea leaves. On the other hand, the formation mechanism of *cis*-4,5-epoxy-(*E*)-2-decenal (**1**) has not yet been clarified. However, it seems likely that the origin and formation mechanism of **1** in the black tea are similar to that of **2**, because, for several black tea cultivars, the contents of **1** have a close relation to that of **2** (**Figure 4**). The formation pathway of **2** formed from linoleic acid by oxidation has already been reported, and two routes (A and B) have been proposed (**Figure 5**). Namely, route A is the pathway in which 2,4-decadienal as the key intermediate reacts with peroxide (*25*), and route B is the pathway in which the 12,13-epoxy-9-hydroperoxy-10-octadecenoic acid decomposes by α-cleavage (*24*). Therefore, in route A, if the other 2,4-alkadienals are included, the corresponding 4,5-epoxy-2-alkenals may also be formed, whereas in route B, 4,5-epoxy-2-decenal could mainly be expected.

Table 2. Amounts of the 2,4-Alkadienals in Black Tea (Dimbula) Infusion

RI[a]	compound	amount (μg/L)
1406	2,4-hexadienal (isomer)	1.36[b]
1414	(*E*,*E*)-2,4-hexadienal	7.72
1471	2,4-heptadienal (isomer)	27.23[b]
1506	(*E*,*E*)-2,4-heptadienal	52.38
1569	2,4-octadienal (isomer)	trace[b]
1601	(*E*,*E*)-2,4-octadienal	0.99
1668	2,4-nonadienal (isomer)	0.12[b]
1712	(*E*,*E*)-2,4-nonadienal	0.87
1770	(*E*,*E*)-2,4-decadienal (isomer)	0.9[b]
1820	(*E*,*E*)-2,4-decadienal	1.76

[a] Retention index in the DB-Wax column observed for GC-MS. [b] These values were estimated by the calibration factor of corresponding 2,4-alkadienals.

The homologous series of 2,4-alkadienals and 4,5-epoxy-2-alkenals in the volatile fraction of the black tea infusion was then screened by GC-MS, and the formation pathway of the epoxydecenal isomers was assumed from both results. In the black tea infusion, the variety of the detected 2,4-alkadienals ranged from six to 10 carbons (**Table 2**). Among these compounds, the 2,4-heptadienals had the highest content, and the contents decreased in the order of the 2,4-hexadienals, then the 2,4-decadienals. However, a considerable amount of the 2,4-octadienals and 2,4-nonadienals were found in the black tea infusion. On the basis of the model experiments, all of the 2,4-alkadienals from six to 10 carbons reacted with the hydroperoxides, which were prepared from linoleic acid with soybean lipoxygenase and produced all of the corresponding 4,5-epoxy-2-alkenals (data not shown). In other words, if the 4,5-epoxy-(*E*)-2-decenal isomers are formed from the 2,4-decadienals as intermediates, it is expected that the homologous series of the 4,5-epoxy-2-alkenals are detected in the black tea. In **Figure 2**, the chromatogram recorded with *m*/*z* 68, which is the typical fragment ion of the homologous series of 4,5-epoxy-2-alkenals, is shown. As a result, only the 4,5-epoxy-2-heptenals and 4,5-epoxy-(*E*)-2-decenals were detected in the black tea, while the other 4,5-epoxy-2-alkenals could not be found. These findings

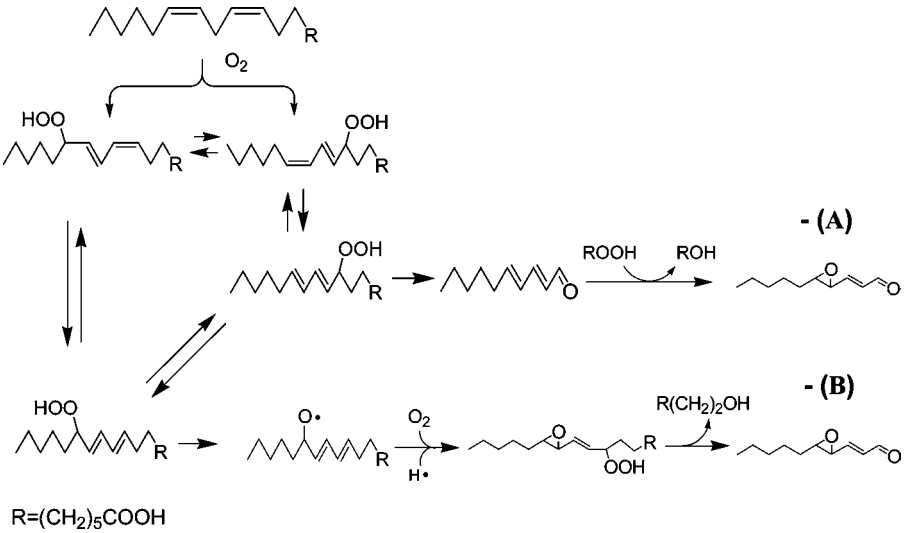

Figure 5. Proposed pathway for the formation of *trans*-4,5-epoxy-(*E*)-2-decenal from linoleic acid [according to Gardner and Selke (*24*) and Gassenmeier and Schieberle (*25*)].

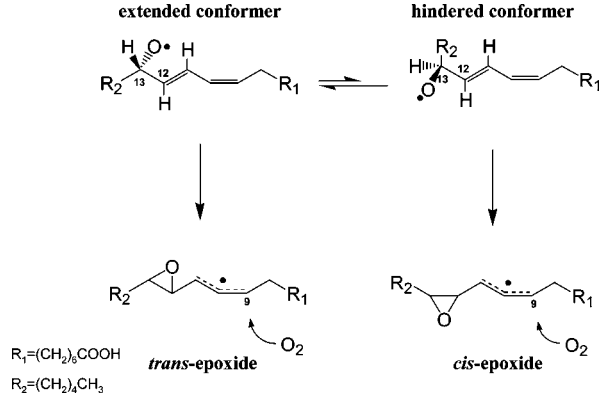

Figure 6. Hypothetical generation mechanism of *cis*- and *trans*-epoxides.

Table 3. Comparison of 4,5-Epoxy-(*E*)-2-decenal Isomers in Black Tea (Dimbula) and Japanese Green Tea (Sen-cha)

	FD factor (4^n)		amount (μg/L)	
odorant	black tea (Dimbula)	green tea (Sen-cha)	black tea (Dimbula)	green tea (Sen-cha)
cis-4,5-epoxy-(*E*)-2-decanal	6	1	0.28	<0.01
trans-4,5-epoxy-(*E*)-2-decaal	7	2	0.60	0.02

suggested that the formation of the 4,5-epoxy-(*E*)-2-decenals cannot be explained by route A via 2,4-decadienal as the intermediate, and it presumed that both isomers in the black tea were indeed formed from linoleic acid in the tea leaves via the 12,13-epoxy-9-hydroperoxy-10-octadecenoic acid.

In addition, the difference in the content of both isomers could be recognized, and the content of **1** was less than that of **2**. This chemical behavior regarding the difference in the contents **1** and **2** can be assumed to affect the equilibration of the alkoxydiene radicals, which are formed from the 13-hydroperoxide of linoleic acid as the intermediate. Namely, the alkoxydiene radicals, which are capable of forming the extended and hindered conformers of the carbon 12,13 bond (**Figure 6**), and the extended conformer are more dominant than the hindered conformer. Therefore, it seems that the content of the *cis*-epoxyallylic radical was less than that of the *trans*-epoxyallylic radical formed from these alkoxydiene radicals. The influence of the equilibrium of the alkoxydiene radical on the formation of the epoxide has already been proposed on the basis of the oxidation mechanism of the lipid (*27*, *28*). Therefore, **1** and **2** in the black tea were expected to be generated from the *cis*- or *trans*-epoxyallylic radicals via the 12,13-epoxy-9-hydroperoxy-10-octadecenoic acid, which oxidized carbon 9 of epoxyallylic radicals with O_2.

The enzyme reactions during the withering and fermentation process were characteristic processes of the black tea production. On the other hand, the green tea leaves inactivated the enzyme by steaming or parching, and it hardly utilizes the action of the enzyme. For the flavor formation of the black tea, in particular, it was pointed out that the enzyme action during the manufacturing process is very important (*10*−*14*). To clarify the relationship of the manufacturing method of the black tea and the formation of the 4,5-epoxy-(*E*)-2-decenal isomers, a comparison of the contents and FD factors of these isomers in the black and green teas was done. It appeared that the FD factor and amount of these odorants in the black tea were much greater in comparison with that of the green tea (**Table 3**). Therefore, **1** and **2** seem to be characteristic compounds for the black tea flavor, and it can be presumed that the black tea production process was very important for the formation of **1** and **2**. The model reactions that simulated the enzyme reaction during the withering and fermentation process of the black tea production were carried out using linoleic acid and its hydroperoxides obtained by the soybean lipoxygenase treatment. As a result, it was shown that **1** and **2** were generated from linoleic acid and its hydroperoxides by heating, and it was observed that the generated amounts of these odorants from the linoleic acid hydroperoxides were much greater than that of linoleic acid (**Table 4**). This finding explained the different amounts of **1** and **2** in the black and green teas and strongly suggested that the enzyme reaction plays an important role in forming **1** and **2** in the black tea.

Table 4. Comparison of Generated Amount of 4,5-Epoxy-(*E*)-2-decenal Isomers in Linoleic Acid and Its Hydroperoxides

	amount (μg)			
	nonheated		heated	
odorant	linoleic acid	LH[a]	linoleic acid	LH[a]
cis-4,5-epoxy-(*E*)-2-decanal	<0.01	<0.01	0.13	2.56
trans-4,5-epoxy-(*E*)-2-decaal	<0.01	<0.01	0.43	6.53

[a] Linoleic acid hydroperoxides.

On the basis of these results, it is suggested that **1** and **2** were generated from linoleic acid contained in the tea leaf during the manufacturing process of the black tea, and it can be assumed that the formation mechanism is as follows. First, the withering and fermentation, which is a characteristic process of the black tea production, converts linoleic acid to its hydroperoxide in which the oxygen adds to the 13 carbon at the *S*- configuration by the lipoxygenase activity, since it is well-known that the tea lipoxygenase has a high regio- and stereospecificity to form the 13(*S*)-hydroperoxide of linoleic acid (*29*, *30*). In addition, by heating during the drying process of the black tea production, the 13(*S*)-hydroperoxide of linoleic acid produced **1** and **2** via the *cis*- and *trans*-epoxyallylic radicals. Therefore, further investigation of the stereochemistry of **1** and **2** in the black tea should serve to clarify the formation mechanism of these odorants. Thus, the formation of **1** and **2** has a close relation to the manufacturing process; especially, the enzyme reaction in the black tea seems to be one of the most important factors for the formation of these isomers.

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